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METHOD FOR CONSTRUCTING LIBRARIES
OF PHENOTYPIC PROFILES

Related Applications

5 This application claims the benefit under 35 U.S.C. §120 or 35 U.S.C. §365(c) of PCT International application PCT/EP99/09710 designating the United States of America, and filed December 7, 1999 of which this application is a national stage filing under 35 U.S.C. §371, was published under PCT Article 21(2) in English, incorporated herein in its entirety by reference.

10 Foreign priority benefits are claimed under 35 U.S.C. §119(a)-(d) or 35 U.S.C. §365(b) of Great Britain application number 9826890.7, filed December 7, 1998, which designated at least one country other than the United States.

Field of the Invention

15 The present invention is concerned with the field of "genetic pharmacology". Specifically, it relates to methods which can determine, among other things, whether a compound has potential pharmacological activity, whether a compound interacts with a particular gene or biochemical pathway in man or animals, what side effects are likely to be associated with a particular pharmaceutical compound and/or the mode or modes

20 of action of any compound with biological activity. Additional uses for the methods of the invention include the assignment of function to particular genes or assignment of genes and their encoded proteins to particular biochemical pathways. In particular, the invention relates to the use of a microscopic nematode worm, for example *Caenorhabditis elegans*, and libraries of such worms in the aforementioned methods.

25 These new methods are able to enhance and accelerate the drug discovery process.

Background

Prior to the early 1990's the search for new compounds having the potential to combat human or animal disease was often begun by taking a compound known to have a particular pharmacological activity, synthesising structurally related variants and then testing those variants against the known target.

The test against the target might be carried out *in vivo*, for example by use of

animal models of a human disease. Alternatively, if a particular molecule as known to be implicated in the progress of a disease, the compounds could be tested for interaction with the molecule *in vitro*. The limitations of such methods are that in the event of a negative result no other information about the pharmaceutical potential of the 5 compound tested is gained. For example, an *in vitro* test might show a compound to have no inhibitory action against a particular target enzyme but that compound might have an inhibitory action against another enzyme in the same biochemical pathway as the target enzyme and therefore, in fact, have potential in treatment of the target disease. Animal tests, while providing a reasonable indication of both efficacy and 10 toxicity, provide no information at all about the mode of action of the compound, and therefore the possible reasons for any toxicity. Furthermore, they are time-consuming and expensive and do not lend themselves to automation.

Since the early nineties there have been two developments in particular which have revolutionized the drug discovery process, these being the new sciences of 15 "genomics" and "combinatorial chemistry". It has now been realised that a vast number of diseases have a genetic component and they are not purely the result of environmental influences. Indeed, it is possible that nearly all diseases are multifactorial and will have some degree of genetic basis, albeit very small in some cases. A huge amount of effort is being directed at the present time to the study of the 20 organisation of the genomes of various unicellular and multicellular organisms, including humans. This involves the identification and sequencing of all the genes in a particular genome. Such activity does not only allow for hunting of genes which are directly associated with particular diseases but each of the genes found and the proteins they encode can become, directly or indirectly, a target against which compounds can 25 be screened, whether or not that gene has yet been associated with a disease or indeed has any identified function at all. Furthermore, rather than starting from a compound of known "activity" and relying on theoretical structure/function relationships to synthesise new candidate compounds, vast libraries of compounds, of uniform activity 30 can be very rapidly synthesized in an automated manner by combinatorial chemistry. Thus, there is now potential to screen thousands of compounds against thousands of genes and the proteins they encode in very rapid high throughput screens (HTS) and to link compounds to genes and genes to disease.

Summary of the Invention

The present inventors have discovered that these new technologies for drug discovery can conveniently be married with a particular multicellular organism, a 5 nematode worm, *C. elegans*, which has been well characterised genetically and morphologically. They have thereby developed new methods, which are extremely powerful, rapid and convenient and can play an essential part in a drug discovery program.

C. elegans is a microscopic nematode worm which occurs naturally in the soil but can be easily grown in the laboratory on nutrient agar inoculated with bacteria, 10 preferably *E. coli*, on which it feeds. Each worm grows from an embryo to an adult worm of about 1 mm long in three days or so. As it is fully transparent at all stages of its life, cell divisions, migrations and differentiation can be seen in live animals. Furthermore, although its anatomy is simple its somatic cells represent most major 15 differentiated tissue type including muscles, neurons, intestine and epidermis. Accordingly, differences in phenotype which represent a departure from that of a wild-type worm are relatively easily observed, either directly by microscopy or by using 20 selective staining procedures, and many of these phenotypic differences submit to quantitative measurement. Many *C. elegans* mutants have been identified and their phenotypes described, for example, see *C. elegans* II Ed. Riddle, Blumenthal, Meyer and Priess, Cold Spring Harbor Laboratory Press, 1997. The *C. elegans* genome is now 25 almost entirely sequenced as a result of the *C. elegans* genome project, carried out at the Sanger Center and Washington University School of Medicine. The sequence is available in a public database at http://www.sanger.ac.uk/projects/C_elegans/. As a result of this it has emerged that *C. elegans* comprises genes which have equivalents that are widely distributed in most or all animals including humans.

Methods for creating mutant worms with mutations in selected *C. elegans* genes are known in the art, for example see J. Sutton and J. Hodgkin in "The Nematode *Caenorhabditis elegans*" Ed. By William B. Wood and the Community of *C. elegans* Researchers CSHL, 1988 594-595; Zwaal et al; "Target-Selected Gene Inactivation in 30 *Caenorhabditis elegans* by using a Frozen Transposon Insertion Mutant Bank" 1993, Proc. Natl. Acad. Sci. USA 90 pp 7431-7435; Fire et al, Potent and Specific Genetic Interference by Double-Stranded RNA in *Caenorhabditis elegans* 1998, Nature 391

860-811.

The possibility that *C. elegans* might be useful for establishing links between compounds and specific *C. elegans* genes by virtue of comparison of phenotypes generated by exposure to particular compounds and by selected mutations is considered
5 by Rand and Johnson in Methods of Cell Biology, Chapter 8, vol 84, *Caenorhabditis elegans: Modern Biological Analysis of an Organism* Ed. Epstein and Shakes, Academic Press, 1995 and J. Ahringer in *Curr. Op. in Gen. & Dev.* 7; 1997; 410-415.

However, these authors observe and attribute altered phenotypes on the basis of a single changed characteristic such as, for example, pharyngeal pumping rate or
10 defecation frequency. Since that single characteristic may be determined by expression of a number of genes and the operation of several biochemical pathways such a crude assessment of phenotype is not sufficient to establish a link between any one gene or pathway and a compound to which the worm has been exposed. As such the procedure would not be sensitive enough for resolution of the properties of thousands of
15 compounds in a high throughput compound screen. An additional problem with the proposals of the prior art is that known phenotypic characteristics have all been described differently by different workers in the *C. elegans* field. Phenotype descriptions in the literature largely omit aspects not directly related to or not recognised to be related to the principle interest of the individual researcher. There is
20 no standard nomenclature to identify a specific change. Without this it is impossible to equate newly observed phenotypes with particular known phenotypes for comparison purposes.

The present inventors have developed methods which solve these problems and thereby have converted *C. elegans* into a really useful tool in the drug discovery field.
25 Specifically, in respect of each worm a "phenotype profile" or "fingerprint" is established based on looking for plurality of changed characteristics in a particular mutant or worm which has been exposed to an environmental change or a compound. Furthermore, each profile is scored by following a strict standard protocol of measurement and a standard description is applied to each characteristic. The
30 determination of a phenotypic profile in this way for a plurality of mutants or worms exposed to compounds illuminates differences between different mutants or otherwise treated worms which would not be apparent based on prior art methods. Furthermore,

the standard scoring protocol and nomenclature allows the phenotypic profiles obtained to be collated into a library of reference profiles for direct comparison purposes. Thus, libraries of reference profiles can be established for mutant worms and for worms exposed to particular environmental changes or different sorts of compounds. Such 5 libraries allow complex patterns of linkage to be established between particular compounds and particular genes or biochemical pathways and between individual compounds of known or unknown biochemical or pharmacological activity.

In accordance with a first aspect of the present invention there is provided a method of constructing a library of phenotypic profiles of nematode worms which 10 comprises the steps of:

- (a) providing a worm having a defect in at least one gene.
- (b) measuring any changes in identifiable characteristics of said worm compared to a worm without said defect,
- (c) systematically scoring a plurality of any said changed characteristics to establish a characteristic phenotype profile associated with said defect,
- (d) simultaneously or sequentially repeating steps (a) to (c) in respect of each of a plurality of worms each of which has a different defect, and
- (e) collating the phenotypic profiles so obtained into a library of said profiles.

20 *Caenorhabditis elegans* is the preferred nematode worm although the method could be carried out with other nematodes and in particular with other microscopic nematodes, preferably microscopic nematodes belonging to the genus *Caenorhabditis*. As used herein the term "microscopic" nematode encompasses nematodes of approximately the same size as *C. elegans*, being of the order 1mm long in the adult 25 stage. Microscopic nematodes of this approximate size are extremely suited for use in mid- to high-throughput screening as they can easily be grown in the wells of a multi-well plate of the type generally used in the art to perform such screening.

It is preferred to establish the phenotypic profile on the basis of the measurement and scoring of at least three different characteristics, preferably at least 30 six characteristics and more preferably at least ten characteristics. It will be appreciated that the more differences which can be scored between a worm with a genetic defect and a worm without the defect the better the resolution between different mutants.

Although not limited to such, at least one of the plurality of changed characteristics which can be measured and scored may be selected from the list shown in Table 1, and possibly each of all the changed characteristics scored is one of those shown in Table 1.

In a preferred embodiment, the method used to establish the phenotypic profile 5 comprises measurement and scoring of two or more characteristics selected from the group consisting of: viability, life cycle, body shape, movement behaviour, mechanotransduction, pharynx pumping, defecation and fertility. This list provides a core set of measurable characteristics which can be used to establish an informative phenotypic profile for any type of worm. Furthermore, each of these characteristics is 10 measurable using technical measuring apparatus, such as video image analysis, multiwell plate reader, and/or a technical assay procedure. In the most preferred embodiment, the method used to establish the phenotypic profile comprises measurement and scoring of all eight of the listed core characteristics. Measuring and 15 scoring this set of core characteristics allows meaningful comparisons to be made between phenotypic profiles for worms subjected to diverse interventions. As exemplified herein, comparisons can be drawn between profiles for two different mutant worms and between profiles for mutant worms and profiles for worms exposed 20 to compound.

It is to be understood the terms "measuring" or "measurement" as used in 25 connection with any of the methods described and claimed herein are to be interpreted as including not just absolute quantitative measurement wherein a numerical value is assigned to the characteristic but also comparative measurement, wherein characteristics of a worm which has been subject to an intervention (i.e. mutation, exposure to compound, exposure to environmental change) are measured relative to the 25 same characteristics of a wild-type worm and scored as being "larger", "smaller", "longer", "shorter", "fatter", "thinner", "darker", "paler" etc.

For comparison purposes it is essential that the scored characteristics are 30 represented in the same order for each profile. For standardization of procedure between different workers or to facilitate automation, measurement and scoring of the characteristics could be carried out in a pre-determined order according to a standard protocol. However, this is not essential to the operation of the method. In its simplest form and as shown in Example 5, the characteristics are recorded in a binary manner as

"present" or "not present" based on deviations from wild-type worms.

It is desirable to establish a library which comprises a phenotypic profile in respect of a defect in each gene in the worm genome and/or different defects in the same gene (allelic variations). As aforesaid there are a considerable number of available mutants (see Riddle, Blumenthal, Meyer and Priess and Ahringer above). In addition new ones can be generated by specific gene and site directed mutation and knock-out methods known to those skilled in the art such as ethyl methanesulphonate (EMS) mutagenesis, transposon insertion or genetic interference using double stranded RNA (see Sutton and Hodgkin, Zwaal et al and Fire et al above). The known or newly generated genetic defects may manifest themselves, for example, as the absence of expression of a gene, the reduction in expression of a gene, the over-expression of a gene, the expression of a functionally defective protein, the mis-expression of a protein, the ectopic mis-expression of a protein, the expression of a protein of altered stability or the alteration of gene expression as a function of time.

Generally, the manipulation of *C. elegans* to generate genetic defects can be carried out on wild-type worms or worms with existing single or multiple mutations. It may be desirable to genetically manipulate *C. elegans* carrying a reporter gene construct. The reporter molecule might be LacZ or green fluorescent protein but many other reporter molecules are known to those skilled in the art. Reporter gene constructs for *C. elegans* are described in Chalfie et al, 1994, *Science* 263 pp 802-805. It can also be desirable to genetically manipulate and then profile a transgenic worm, preferably a worm carrying a human gene, particularly where the gene is associated with, or is a candidate for association with a human disease and therefore a putative drug target. A list of human diseases for which a particular gene has been implicated is given in the paper by J. Ahringer (see above) and also provided by OMIM. Center for Medical Genetics, John Hopkins University and National Biotechnology Information, National Library of Medicine, 1996. <http://www.ncbi.nlm.nih.gov/omim/>, although these lists are not necessarily exhaustive.

It is easy to establish transgenic lines in *C. elegans* and the methodology is described in Craig Mello and Andrew Fire, *Methods in Cell Biology*, Vol 48 Ed. H.F. Epstein and D.C. Shakes, Academic Press, pages 452-480.

A form of the worm which may show a change in phenotype and may therefore

be subject to profiling as described above is one in which the genetic defect and/or transgene and/or reporter gene is only present in a sub-set of the cells of the worm. It is possible for just the cells of a particular tissue to be the subject of a genetic manipulation.

5 The worm which is to be subject to determination of its phenotypic profile can be cultured by methods well-known in the art. *C. elegans* can grow on nutrient agar which has first been inoculated with bacteria on which the worms feed. Suitable culture methods are described in Rand and Johnson (see above) and in the examples given herein. Measurement of any changed characteristics which will determine the profile
10 may be carried out using light microscopy, differential interference contrast optics or fluorescence microscopy. In addition immuno-chemical detection, colorimetric detection or detection of fluorescence, luminescence or radioactive labels may be used. In some cases the changed characteristics may be biochemical only and might be detected, for example by a pH change in the growth media or a change in electrical
15 potential. Different characteristics may need to be determined at different points in the growth cycle of the worm. For example, some phenotypic characteristics may be manifested only in the larvae while others are only detectable in the adult worm. In some cases it may be necessary to make several measurements of the same characteristic at pre-determined time intervals.

20 Phenotypic profiles generated by the methods described above can be collated into a library of profiles which are stored electronically on a database. However, it will be appreciated that the invention also provides a method of constructing a physical library or bank of worms each identifiable by their individual phenotypic profile. Such a worm library can be created using any or all of the methods described above and used
25 for comparative purposes. The worms may be maintained by the culture methods described herein and/or frozen for long term storage by methods known to those skilled in the art. Libraries of phenotypic profiles or fingerprints of mutant worms or mutant worm libraries can be used to determine linkages between different genes and hence identify biochemical pathways. A particularly important use is the profiling of several
30 mutations of the same gene and several genes of the same pathway. Different mutations in the same gene can have different phenotypes and often it is found that a careful analysis of the allelic series of a gene reveals important information that is hidden

under a more severe phenotype of a null mutant (complete knock out, e.g. if it is lethal). Phenotypic profiles of different mutations of the same gene allow characterisation of the gene by simply combining (logical OR) the profiles of all the mutations, whether they have been generated at the same time or not. It is possible, however, to handle the 5 mutations separately and make more detailed connections, for example, concerning protein domains in case the similarity of phenotypes cluster with the sites of the mutations.

Described above are methods for constructing a library of phenotypic profiles for worms with a plurality of genetic defects or a library of mutant worms. However, in 10 accordance with a second aspect the present invention provides a method of constructing a library of phenotypic profiles of nematode worms which comprises the 15 steps of:

- (a) exposing a worm to a compound,
- (b) measuring any changes in identifiable characteristics of said worm as a 15 result of exposure to said compound,
- (c) systematically scoring a plurality of any said changed characteristics to establish a phenotypic profile associated with said compound,
- (d) simultaneously or sequentially repeating steps (a) to (c) in respect of each of a plurality of different compounds, and
- 20 (e) collating the phenotypic profiles so obtained into a library of said profiles.

Methods for culturing *C. elegans* in the presence of a test compound are described by Rand and Johnson mentioned above and in the examples herein. In its simplest form a solution of the compound in a suitable solvent may be spread over a 25 bacterial lawn on an agar plate before inoculation with the worm. Additional refinements include feeding the worm with bacteria, preferably *E. coli*, which have taken up the compound or attaching the compound to a carrier compound which is particularly attractive to the worm.

The worms which are exposed to the compound may be wild-type worms, 30 mutant worms, transgenic worms and/or worms carrying reporter gene constructs as already described herein. Further the measurement and scoring of a plurality of changed characteristics is carried out by exactly the same procedures as already described herein

for the phenotypic profiling of mutant worms. This must be a standard format in order that direct comparisons can be made between profiles obtained on exposure to compounds and profiles exhibited by mutants.

With compound screening it is possible to build up a series of different libraries depending on the compounds being tested. For example one library can comprise profiles generated in respect of each of the known compounds in a Pharmacopoeia, in other words compounds with known pharmacological activity.

Another library can comprise profiles generated by compounds known to interact with a particular biochemical pathway, which may or may overlap with those compounds from the Pharmacopoeia. Other libraries could include profiles for known compounds but with no known biological activity or compounds which are completely new molecules such as might be generated by combinatorial chemistry. As aforesaid the present invention is not limited to the production of phenotypic profile libraries but includes libraries or banks of worms whose phenotypic profile has been altered by exposure to compounds. In particular embodiments assays may be carried out with several concentrations of the same compound, and/or with mixtures of compounds. For example compounds from compound libraries may each be tested individually or with one or more other influencing compounds. Furthermore, such compound testing protocols may be executed against identical worms or multiple mutant and/or transgenic backgrounds. In a particular example a panel of worm strains, covering a wide range of biochemical pathways and cellular activities by means of mutations in particular pathways, as well as reporter genes, is used for testing compounds. For each compound, potentially at several concentrations, a profile is recorded for the measurable phenotypes of each of the worm strains, either in parallel or sequentially.

In a third of its aspects the invention provides a method of constructing a library of phenotypic profiles of nematode worms which comprises the steps of:

- (a) exposing a worm to an environmental change,
- (b) measuring any changes in identifiable characteristics as a result of said environmental change,
- (c) systematically scoring a plurality of any said changed characteristics to establish a characteristic phenotypic profile associated with said change,

- (d) simultaneously or sequentially repeating steps (a) to (c) for each of a plurality of different environmental changes, and
- (e) collating the phenotype profiles so obtained into a library of said profiles.

5 The environmental change may be, for example, a change in pH, osmolarity, temperature, exposure to radiation or exposure to bacteria or viruses. Each of these external influences may result in the manifestation of a different phenotypic profile of characteristics so that libraries of said profiles and affected worms can be constructed. Again, measurements and scoring of the profile should follow a standard protocol in
10 order that valid comparisons can be made between these profiles and those in mutant and compound libraries.

The construction of worm and phenotypic profile libraries by the methods described above using the novel phenotypic profiling method described herein provides a very powerful tool for the discovery of new drugs. Profiles in each of the different
15 libraries can be compared and links established between *C. elegans* genes and pathways, compounds and environmental effects. Preferably, the process of measuring and scoring the changed characteristics which go to make up the phenotypic profile is automated, making use of technical measuring apparatus. The profiles so generated may advantageously be stored electronically. Libraries of profiles can then be searched by computer which can identify identical or similar profiles, either within or between
20 the different libraries. Quantitative data calculations, optionally in combination with boolean operations can be used.

A comparison of the profile generated by a particular compound with the profiles of particular mutants may indicate the likely gene or biochemical pathway with
25 which the compound interacts in the worm. Other databases can then be searched for a match of the worm gene with an equivalent human gene. The human gene might already be associated with a human disease as could be determined for example, from the OMIM database mentioned above. Thus, by use of the worm screen a potential candidate drug can be identified. The discovery of the mode of action of a compound
30 with known pharmacological or biochemical activity is facilitated by comparing its phenotypic profile in the worm with the mutant library or environmental change library of profiles to identify possible targets for the compound. other possibilities include

finding a new potential medical indication of a known compound, a medical indication for a novel compound, an alternative method of treatment of a known disease or an indication of the reason for the side effect exhibited by some known pharmaceuticals. Testing worms with compounds, scoring the phenotypic profile in the novel manner 5 described herein and then searching previously established libraries of profiles can potentially achieve all those goals. Once a compound has been identified as having the potential to be a therapeutic agent it can be processed through the more traditional drug discovery routes. The compound can be tested in more specific in vitro tests based on the new knowledge of the target for the compound and in animal models of the target 10 disease. Structural variants then can be generated by medicinal chemistry with a view to improving activity.

The invention will now be described with reference to the following Examples, together with accompanying Figures, in which:

15 **Brief Description of the Drawings**

Figure 1 is a schematic diagram of the left lateral view of the body of *C. elegans*. The body of *C. elegans* is divided into a head, a body and a tail region. The head region stops at the end of the pharynx, the body stops at the rectum and the tail includes the tail whipe. *C. elegans* usually crawl on the right side. The ventral located 20 vulva defines the ventral side of *C. elegans*.

Figure 2 is a schematic diagram of *C. elegans* showing the characteristics “hypertrophy of the head” and “extensions on head”.

Detailed Description

25 **Example 1**

General Profiling by Plate Drop Assay

4m1 NGM agar (see “The Nematode Caenorhabditis Elegans” Ed. by William B. Wood and the Community of *C. elegans* Researchers CSHL, 1988, pg 589) is poured into 3cm plate, and seeded with approximately 5 μ l of an *E. coli* overnight culture and 30 grown preferably for one week at room temperature. If a compound is to be profiled 10 μ l of compound dissolved in DMSO or other appropriate solution is pipetted onto the bacterial lawn. The lawn should be covered completely. (This step can be omitted if

a mutant, transgenic or other worm is being profiled without compound). After overnight soaking in of compound one C. elegans (L4 stage) per plate is put in the bacterial lawn. Worms are checked after some hours, plates are incubated at 21°C and worms screened for phenotypes (control have L1 progeny growing). Plates are checked
5 again after 4 days for phenotypes of F1 progeny (control shows all stages up to gravid hermaphrodites). Plates which have to be looked at again on subsequent days because of slow growth or for further checks are put aside. A plate protocol sheet such as that shown in Table 2 is completed deciding on one of the following routes: no effect/unspecific effect/needs to be applied at lower concentrations/needs to be profiled.
10 If concentrations are appropriate and a decision can be made scoring of characteristics to produce a profile can be started using the profiling list in Table 1. Because the compound is pipetted onto a bacterial lawn rather than it being incorporated into the agar, as has been done in the prior art, this method is designated a “plate drop assay”.

15 **Table 1**

1. Compound specific phenotypes

Phenotype							Comment
1.1 Disappeared							
1.2 Determining compound action							
1.2.1 acute death without tracks							
1.2.2 acute death with tracks							
1.2.3 burst							
1.2.4 dissolving							
1.2.5 pale							
1.3 Compound response							
1.3.1 tracks not in center							
1.3.2 tracks inside							
1.3.3 tracks more outside							
1.3.4 tracks only outside							
1.3.5 tracks invisible							
1.3.6 attraction							
1.3.7 avoidance (try to avoid)							
1.3.8 avoidance (try to escape)							
1.4 Course of compound response							
1.4.1 immediate response							
1.4.2 delayed response							
1.4.3 progression of phenotype							
1.4.4 shift of phenotype							
1.4.5 recovered from exposure							
1.4.5.1 compound inactive							
1.4.5.2 irreversible							
1.4.5.3 adapted to compound							
1.5 Later exposed worm different							
1.5.1 weaker							

1.5.2 worse						
1.5.3 lower penetrance						
1.5.4 higher penetrance						
1.5.5 not affected						

2. Viability

Phenotype						Comment
Abnormal						
2.1 Dead adult (P0; during 3 days)						
2.2. Partial lethality						
2.2.1 Few dead eggs						
2.2.2 Few dead larvae						
2.3 Embryonic arrest of F1						
2.3.1 Leaky ness						
2.3.2 Appearance of eggs						
2.3.2.1 <i>dark eggs</i>						
2.3.2.2 <i>bright eggs</i>						
2.3.2.3 <i>two-fold or older</i>						
2.3.2.4 <i>irregular egg size</i>						
2.4 Larval arrest of F1						
2.4.1 Leaky ness						
2.4.2 at L1						
2.4.3 at L2						
2.4.4 at L3						
2.4.5 at L4						
2.5 Embryonic arrest of F2						
2.5.1 Leaky ness						
2.5.2 Appearance of eggs						
2.5.2.1 <i>irregular egg size</i>						
2.6 Larval arrest of F2						
2.6.1 Leaky ness						
2.6.2 at L1						
2.7 Died during adulthood (F1)						
2.8 Died during adulthood (F2)						

3. Life cycle

Phenotype						Comment
Abnormal						
3.1 Growth abnormal						
3.1.1 only generation cycle slowed down						
3.1.1.1 <i>oldest stage L1</i>						
3.1.1.2 <i>oldest stage L2</i>						
3.1.1.3 <i>oldest stage L3</i>						
3.1.1.4 <i>oldest stage L4</i>						
3.1.2 generation cycle slowed down while displaying defects						
3.1.2.1 <i>oldest stage L1</i>						
3.1.2.2 <i>oldest stage L2</i>						
3.1.2.3 <i>oldest stage L3</i>						
3.1.2.4 <i>oldest stage L4</i>						
3.1.3 stage changed						
3.1.3.1 <i>delayed hatching</i>						

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3.1.3.2 arrested growth in L1						
3.2 Dauer formation defective						
3.2.1 constitutive dauer						
3.2.2 non-conditional constitutive						
3.2.3 defective						
3.2.4 dies on recovery						
3.3 Life span changed						
3.3.1 Life span is shorter						
3.3.2 Life span is prolonged						

4. Body shape

Phenotype						Comment
Abnormal						
4.1 Proportion abnormal						
4.1.1 short						
4.1.2 long						
4.1.3 thin						
4.1.4 thick						
4.1.5 small (short and thin)						
4.1.6 large (long and thick)						
4.1.7 dumpy						
4.1.7.1 <i>piggy</i>						
4.1.7.2 <i>lumpy</i>						
4.1.7.3 <i>weak (umpyish)</i>						
4.1.7.4 <i>medium</i>						
4.1.7.5 <i>strong</i>						
4.2 Head defects						
4.2.1 extensions, protrusions						
4.2.2 hypertrophy						
4.2.2.1 <i>hypertrophy ventral side</i>						
4.2.2.2 <i>hypertrophy dorsal side</i>						
4.2.2.3 <i>hypertrophy left side</i>						
4.2.2.4 <i>hypertrophy right side</i>						
4.2.3 dystrophy						
4.2.3.1 <i>dystrophy ventral side</i>						
4.2.3.2 <i>dystrophy dorsal side</i>						
4.2.3.3 <i>dystrophy left side</i>						
4.2.3.4 <i>dystrophy right side</i>						
4.2.4 only head bent						
4.2.5 hammer head						
4.2.6 swollen						
4.2.7 rounded						
4.2.8 short and rounded						
4.2.9 tapering						
4.2.10 notched						
4.2.11 vacuoles only in head						
4.2.12 autodecapitation						
4.3 Body defects						
4.3.1 bent body						
4.3.2 U-shaped						
4.3.3 humpback (dorsal lumps)						
4.3.4 truncated						
4.3.5 withered						

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4.3.6	twisted							
4.3.7	spindle-shaped							
4.3.8	scrawny							
4.3.9	fat							
4.3.10	pale							
4.3.11	pale with dark spots							
4.3.12	clear							
4.3.13	extensions, protrusions							
4.3.14	fluid-filled							
4.3.15	full of vacuoles							
4.4	Tail defects							
4.4.1	only tail truncated							
4.4.2	knob-like							
4.4.3	tapering							
4.4.4	only tail withered							
4.5	Cuticle defects							
4.5.1	blistered							
4.5.1.1	<i>symmetrically</i>							
4.5.1.2	<i>around the head</i>							
4.5.1.3	<i>around the pharynx</i>							
4.5.1.4	<i>around the body</i>							
4.5.1.5	<i>around the tail</i>							
4.5.2	moultling defective							
4.5.2.1	<i>incomplete molts</i>							
4.5.2.2	<i>supernumerary molts</i>							
4.5.3	burst							
4.6	Poured out							

5. Movement

Phenotype					Comment
Abnormal					
5.1 No movement/Motionless					
5.1.1 stiff rods					
5.1.2 loose rods					
5.1.3 lay still					
5.1.4 completely stretched out					
5.1.5 clenched					
5.1.6 jerky					
5.1.7 wiggle					
5.1.8 omega appearance					
5.1.9 capital omega appearance					
5.1.10 straight but head motion					
5.1.10.1 sniffling					
5.1.10.2 reduced head motion					
5.1.11 coiler					
5.1.11.1 tends to coil					
5.1.11.2 weak coiler					
5.1.11.3 strong coiler					

5.1.11.4 <i>vulva always outside</i>							
5.1.11.5 <i>vulva always inside</i>							
5.1.11.6 <i>simultaneously folding in both the anterior & the posterior parts</i>							
5.1.11.7 <i>spiralling inwards anteriorly</i>							
5.1.11.8 <i>spiralling inwards posteriorly</i>							
5.2 Slow movement							
5.3 Enhanced movement							
5.4 Irregular movement							
5.4.1 shaker							
5.4.2 erratic							
5.4.3 curly							
5.4.4 jerky movement							
5.4.5 weak kinker							
5.4.6 strong kinker							
5.4.7 preferred direction							
5.4.7.1 <i>moves better forward</i>							
5.4.7.2 <i>moves better backward</i>							
5.4.7.3 <i>moves always forward</i>							
5.4.7.4 <i>moves more often backward</i>							
5.4.8 loopy movement							
5.4.9 rolling							
5.4.9.1 <i>right-handed</i>							
5.4.9.2 <i>left-handed</i>							
5.4.10 spinning round							
5.4.10.1 <i>in a circle</i>							
5.4.10.2 <i>in a curled circle</i>							
5.4.11 kicker							
5.4.12 twitcher							
5.4.13 amplitude increased							
5.4.14 amplitude decreased							
5.4.15 amplitude weak exhibited							
5.4.16 body is dragged by head							
5.5 Head movement abnormal							
5.5.1 loopy head movement							
5.5.2 head movement reduced							
5.5.3 head movement enhanced							
5.6 Tail movement abnormal							
5.6.1 clenched							
5.6.2 tail is dragged by body							

6. M chanotransduction (Touch with a wire and with ey lash)

Phenotype							Comment
6.1 Harsh touch response abnormal							
6.1.1 no plate drop response							
6.1.2 no movement							
6.1.3 irregular movement							
6.1.3.1 <i>moves not forward</i>							
6.1.3.2 <i>moves forward abnormal</i>							
6.1.3.3 <i>moves not backward</i>							
6.1.3.4 <i>moves backward abnormal</i>							
6.1.3.5 <i>moves better forward</i>							
6.1.3.6 <i>moves better backward</i>							
6.1.4 cramped before movement							
6.1.5 shrinker before movement							
6.2 Harsh touch reflex abnormal							
6.2.1 no plate drop reflex							
6.2.2 movement after prodding							
6.2.2.1 <i>sleepy</i>							
6.2.3 no reflex							
6.2.4 irregular reflex							
6.2.4.1 <i>no move back reflex</i>							
6.2.4.2 <i>weak move back after reflex</i>							
6.2.4.3 <i>no move forward reflex</i>							
6.2.4.4 <i>weak move forward reflex</i>							
6.2.5 cramped							
6.2.6 shrinker							
6.3 Nose touch avoidance abnormal							
6.3.1							
6.4 Foraging behaviour abnormal							
6.4.1							
6.5 Body touch response abnormal							
6.5.1							

7. Sensory system

Phenotype							Comment
Abnormal							
7.1 Avoidance of bacteria							
7.2 Bordering behaviour							
7.3 Chemotaxis defective							
7.3.1 attraction							
7.3.2 avoidance							
7.4 Thermotaxis defective							

7.4.1 attraction							
7.4.2 avoidance							

8. Environmental response

Phenotype							Comment
Abnormal							
8.1 Osmolarity sensitive							
8.2 Thermotolerance changed							
8.3 UV Resistance changed							
8.4 Oxygen sensitive							

9. Pharynx

Phenotype							Comment
Abnormal							
9.1 Pharynx stuffed							
9.2 Morphology defects							
9.3 Pumping defects							
9.3.1 pumping reduced							
9.3.2 pumping enhanced							
9.3.3 pumping irregular							
9.3.4 no pumping							
9.4 Eating defective							

5

10. Intestine

Phenotype							Comment
Abnormal							
10.1 Morphology defects							
10.1.1 enlarged							
10.1.2 detached							
10.2 Color of contents							
10.2.1 darker							
10.2.2 lighter							

11. Rectum

Phenotype							Comment
Abnormal							
11.1 Morphology defects							
11.1.1 protruding							

11.1.2 scarring						
11.1.3 absent						
11.2 Constipation						
11.2.1 foregut filled/enlarged						
11.2.2 hindgut weak						
11.2.3 hindgut strong						
11.3 Defecation cycle defective						
11.3.1 expulsion defective						
11.3.1.1 <i>weak expulsion</i>						
11.3.1.2 <i>no expulsion</i>						
11.3.2 aBoc defective						
11.3.3 pBoc defective						
11.3.4 wrong timing of cycle						

12. Gonad

Phenotype						Comment
Abnormal						
12.1 Morphology defects						
12.1.1 defective gonad						
12.1.2 one arm missing						
12.1.3 multiple gonad						
12.1.4 monopolar gonad forward						
12.1.5 monopolar gonad backward						
12.1.6 no gonad						
12.2 Light brown						

13. Vulva

Phenotype						Comment
Abnormal						
13.1 Morphology defects						
13.1.1 defective vulva						
13.1.2 protruding vulva						
13.1.3 multi vulva (number)						
13.1.4 no vulva						
13.1.5 leaky vulva						
13.1.6						
13.1.7						

14. Fertility

Phenotype						Comment
Abnormal						
14.1 Brood size abnormal						
14.1.1 smaller						
14.1.2 larger						
14.2 Egg laying defect						
14.2.1 no egg retention						
14.2.2 immediate Egl						
14.2.3 progressive Egl						
14.2.4 egg laying defective						
14.2.4.1 <i>weak Egl</i>						
14.2.4.2 <i>strong Egl</i>						
14.2.5 bloated worms						
14.2.5.1 <i>weak bloating</i>						
14.2.5.2 <i>strong bloating</i>						
14.2.5.3 <i>bags of worms</i>						
14.2.6 no egg laying						
14.3 Only oocytes						
14.4 Sterile						
14.5 Maternal effect sterile						

15. Male

Phenotype						Comment
Abnormal						
15.1 Frequency						
15.1.1 high incidence of males						
15.2 Mating defective						
15.3 Morphology						
15.3.1 leptoderan tail						
15.3.2 scrawny						
15.3.3 copulatory plug						
15.4 Mating behaviour						
15.4.1 defective sensory contact						
15.4.1.1 <i>no response to dorsal contact</i>						
15.4.1.2 <i>no response to ventral contact</i>						
15.4.2 defective backing						
15.4.2.1 <i>no backing</i>						
15.4.2.2 <i>no continued backing</i>						
15.4.3 defective turning						
15.4.3.1 <i>loose turns</i>						
15.4.3.2 <i>stop at the tail</i>						

15.4.3.3 slide off the tail						
15.4.4 defective vulval location						
15.4.5 defective spicule insertion						

16. Progression of phenotype

Phenotype						Comment
Abnormal						
16.1 Dependent on generation						
16.1.1 F1 different from P0						
16.1.1.1 weaker						
16.1.1.2 worse						
16.1.1.3 lower penetrance						
16.1.1.4 higher penetrance						
16.1.1.5 not affected						
16.1.2 F1 different from F2						
16.2 Dependent on stage						
16.2.1 appearance of phenotype						
16.2.1.1 after L2						
16.2.1.2 during adulthood						
16.2.2 shift of phenotype						
16.3 Dependent on age						
16.3.1 phenotype gets worse						
16.3.2 phenotype gets better						

Table 2

plate	well	by	date
negative control	positive control	Finished	confirmed (_3 worms)
no effect	unspecific effect	needs to be applied at lower concentrations	needs to be profiled

5

Day 0

compound
invisible
coloured
droplets
crystals
complete crust

bacteria
normal lawn
grown as ring
thin
crust
died

worm
happy
run away
irregular movement
slow movement
no movement

Day 1

appearance
healthy
slightly unhealthy
slightly starved
strong starved
very sick

worm gone
lost
suicide
in agar
starved outside
died in compound

replaced by
number and stage
left progeny

10

movement
normal
tracks more outside

body
normal gravid adult
pumping defects

progeny
normal
reduced broodsize

tracks not in center
amplitude increased loopy
amplitude variable
amplitude decreased
enhanced movement
slow movement
no movement
specific

light brown messy gonad
pale with dark spots
few eggs in gonad
pharynx stuffed
foregut filled large
hindgut constipated
protruding vulva
other:

younger staged
oocytes
coagulated eggs
dead eggs
dying hatchlings
crippled larvae

Day 4

food
still plenty of
already finished
finished soon
outside comp.
not eatable, died
movement
normal
population more outside
population not in center
amplitude increase, loopy
amplitude variable
amplitude decreased
enhanced movement
slow movement
no movement
specific:

adult viability
still fertile
laying oocytes
died
died as bag of worms
missing
body
normal gravid adult
pumping defects
light brown messy gonad
pale with dark spots
few eggs in gonad
pharynx stuffed
foregut filled large
hindgut constipated
protruding vulva
other:

growth rate
normal
reduced broodsize
younger staged
brood viability
dead eggs
dead larvae
larval arrest
later scoring
day of screen
day of worm

comparison of phenotypes

progeny shows PC phenotype
similar
worse
a few only
weaker
no effect

new worms show phenotype
similar
worse
not all
weaker
not effect

stage & age
all stages
young only
late larvae and adults
adults only
old adults

5

comparison to other plates

comparison to known drugs

comparison to known mutants

Example 2

Profiling of a compound library (new compounds)

10 To profile new compounds from a library, the general profiling protocol is followed with the variations.

Compounds are profiled once in undiluted concentration, the actual concentration being dependent on the compound library in question but will be between 0.01 mg and 1 mg of compound/10 μ l DMSO.

15 For compounds with a MW of 500 this calculates to 2-200 mM stock. Dilution in 4m1 agar would be at 5-500 μ M. The high dose may create lots of unspecific effect problems e.g. bacterial death and worm starvation. Thus, if necessary the compounds are applied in a second round at lower concentrations which are dilutions in DMSO of 1/3, 1/10 and 1/30 of the undiluted concentration. A concentration is finally chosen for each compound which will allow a phenotype profile to be established according to the

5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

standard procedure.

Example 3

Profiling of known compounds (biotools, pharmacopoeia)

5 To profile known compounds from a library the general profiling protocol is followed with the following variations. The stock solution is preferred as 100mM in DMSO and the experiment is started *ab initio* with a concentration series. The concentration series is used as described below. In one series of concentrations 15 or so worms (for a reasonable number of short term effects) are placed in the agar. In three 10 series 1 worm each is placed on the agar to score a reasonable number of progeny. Lost worms of the latter three series of concentrations can be replaced from the large pool where worms have been exposed to the compound in the same way. The following concentrations can be used:

conc.in 10 μ l drop	100mM	30mM	10mM	3 mM	1mM	0.3mM
conc.in 4ml drop	100 μ M	300 μ M	100 μ M	30 μ M	10 μ M	μ 3 μ M

15

Example 4

Comparison of agar assay to drop assay

A set of compounds from the pharmacopoeia have been profiled using the general protocol (all compounds were of known activity and are described in 20 Martindale: The Complete Drug Reference, 32nd edition, Pharmaceutical Press 1999). The plate drop assay was compared against standard of pouring compounds into the agar as described in literature which method is designated agar assay. In the drop assay as well as in the agar assay, the compounds were added to the worm in a variety of concentrations, and the survival of the worm was scored as well as the phenotypic 25 profile induced by the compound. The lowest concentration of a compound, still resulting in the death of the nematode was designated minimal lethal dose. The maximal concentration of a compound that did not result in the death of the nematode was designated maximal nonlethal dose. The minimal concentration of a compound that still resulted in a measurable phenotype was designated minimal effective dose. The 30 concentrations of the compounds in the agar assay were compared to the concentrations

in the drop assay. From this observation one may conclude that the newly described drop assay protocol turns out to be far more efficient for most compounds. The following table lists the calculated concentration ratio needed to get the same effect with the compound in the agar assay (in 2 ml agar) rather than the drop assay (in 4 ml agar).

Table 3

Compound	Site	min. lethal dose	max. nonlethal dose	min. effective dose	Average potency ratio
Ketanserine	serotonin rec. agonist	>610			610
Tamoxifen	estrogen rec. antagonist	204	304		254
Fluoxetine	serotonin reuptake inh.	124	186		154
Pancuronium	nicotinic antagonist			>100	100
Methoxyphenylpiperazin	α -adrenorec. ligand	>48	>146	72	88
Naloxone	opioid antagonist		>44	78	60
Diheptylbipyridinium	ryanodine rec. antag.	20	30	36	28
W7	calmoduline antag.	20		10	14
Thapsigargin	serca antagonist				14
Physostigmine	cholinesterase inh.			8	8
Lobeline	nicotinic rec. ligand			4	4
Riluzole	glutamase release inh.	2	2	4	2
Levamisole	acetylch. rec. antag			$\frac{1}{2}$	$\frac{1}{2}$
Nicotine	acetylch. rec. antag			$\frac{1}{2}$	$\frac{1}{2}$

Minimal lethal dose: rate between the lowest concentration in which the compound is lethal to the worm in both assays Maximal non-lethal dose: rate between the highest concentration in which the compound is not lethal in both assays.

Minimal effective dose: rate between the lowest concentration in which the compounds results in a phenotype in both assays.

Average: average of the rates.

15

Example 5

Preferred set of informative characteristics

Worms exposed to a compound, carrying a mutation or are transgenic are examined for the following 8 informative features/phenotypes:

1. **Viability**

Worms are examined for viability at all stages of the life cycle, being embryogenesis, larval stages 1 to 4 and adulthood. Dead embryos are defined by not hatching within 24h and dead worms are defined by not moving, by lack of pharynx pumping, by sick or pale appearance and by lack of response to mechanical stimulation.

Method:

Embryonic lethality is measured by counting the amount of unhatched worms after 24 hours (Elispot, Zeiss). Counting of unhatched worms could also be automated using the FANS device, described below. Viability of larvae and adults is measured by dye uptake.

2. **Life cycle**

Progeny are examined for the length of the generation cycle in comparison to control progeny (of a wild-type worm). The stage of a synchronized progeny will be compared to the stage of a synchronized control progeny (N2, Bristol strain) after three days at 20°C. The developmental stages can be distinguished by vulva development, expression of stage-specific markers, such as collagen IV, body length and transparency.

Method:

Measuring the body length of a population allows determination of the actual stage in the life cycle (For body shape measurement, see 3. Body shape). Expression of stage-specific markers can be examined using antibodies of the appropriate specificity, by way of example an antibody that recognizes an antigen on the surface of *C. elegans* L1 larvae has been described by Hemmer *et al.*, (1991) *J Cell Biol*, 115(5): 1237-47.

3. **Body shape**

Worm size is determined by measuring worm length and worm diameter.

Method:

The body length of a synchronized progeny of adult worms is compared to the body length of a synchronized control progeny (N2, Bristol strain). Measurement of

body length can be achieved using a “worm dispenser apparatus” which is commercially available from Union Biometrica, Inc, Somerville, MA, USA. This apparatus has properties analogous to flow cytometers, such as fluorescence activated cell scanning and sorting devices (FACS). Accordingly, it may be commonly referred 5 to as a “FANS” apparatus, for fluorescence activated nematode scanning and sorting device (FANS). The FANS device enables the measurement of properties of microscopic nematodes, such as size, optical density, fluorescence, and luminescence.

10 Body size may also be measured via image analysis, in which case the measurements recorded may include worm diameter and deviation from the typical tube shape of a wild-type worm.

4. Movement behaviour

The measurement of movement behaviour can include measurement of the speed of movement, or of the pattern of movement (e.g. direction) or both. A wild-type worm moves in a sinusoidal way forward and pauses or moves backward occasionally. 15 Any deviation from this wild-type pattern of movement can be scored as a “changed” characteristic.

Method:

20 An assay based on the following principles may be used to determine the speed of movement of a worm culture:

Nematode worms that are placed in liquid culture will move in such a way that they maintain a more or less even (or homogeneous) distribution throughout the culture. Nematode worms that are defective in movement will precipitate to the bottom 25 in liquid culture. Due to this characteristic of nematode worms as result of their movement phenotype, it is possible to monitor and detect the difference between nematode worms that move and nematodes that do not move.

Advanced multi-well plate readers are able to detect sub-regions of the wells of multi-well plates. By using these plate readers it is possible to take measurements in 30 selected areas of the surface of the wells of the multi-well plates. If the area of measurement is centralized, so that only the middle of the well is measured, a difference in nematode autofluorescence (fluorescence which occurs in the absence of

any external marker molecule) can be observed in the wells containing nematodes that move normally as compared to wells containing nematodes that are defective for movement. For the wells containing the nematodes that move normally, a low level of autofluorescence will be observed, whilst a high level of autofluorescence can be observed in the wells that contain the nematodes that are defective in movement.

In an adaptation of the movement assay, autofluorescence measurements can be taken in two areas of the surface of the well, one measurement in the centre of the well, and one measurement on the edge of the well. Comparing the two measurements gives analogous results as in the case if only the centre of the well is measured but the additional measurement of the edge of the well results in an extra control and somewhat more distinct results.

As an alternative to the above-described movement assay, specialist software such as SIMI Scout (designed for movement study of an athlete) may be used to determine speed of movement, deviation from sinusoidal movement and even the overall pattern of movement of the worm.

5. **Mechanotransduction**

Worms are examined for response to mechanical stimulation.

20 Method:

When the plate on which *C. elegans* are cultured is dropped wild-type worms react by enhanced movement and enhanced overall activity. The capability of a worm to respond to a mechanical stimulus is measured by the difference in speed of movement before and after stimulation.

25

6. **Pharynx pumping**

The phenotypes "Pumping frequency reduced, Pharynx pumping irregular" etc. describe the activity of the cyclic contraction of the pharynx muscles that occurs in a feeding adult about 3 times in a second. The contraction cycle can be described as the nearly simultaneously contraction of the corpus, anterior isthmus, and terminal bulb, followed by relaxation.

Method:

The following pharynx pumping characteristics may be analyzed by image analysis: The frequency of pumping by counting the pharynx contraction. Pharynx contraction can be measured visibly by the opening and closing of the anterior corpus.

5 The time of opened anterior corpus and the diameter of the opened corpus is used to measure hypercontraction, relaxation and strength of a contraction.

The following is an example of a pumping assay which allows measurement of the total efficiency of feeding of a worm, which is related to pumping:

10 The pumping rate of the pharynx is measured indirectly by adding a marker molecule precursor such as calcein-AM to the medium and measuring the formation of marker dye in the *C. elegans* gut. Calcein-AM is cleaved by esterases present in the *C. elegans* gut to release calcein, which is a fluorescent molecule. The pumping rate of the pharynx will determine how much medium will enter the gut of the worm, and hence how much calcein-AM will enter the gut of the worm. Therefore by measuring the 15 accumulation of calcein in the nematode gut, detectable by fluorescence, it is possible to determine the pumping rate of the pharynx.

20 To perform the pharynx pumping screen with calcein-AM, a concentration of between 1 and 100 μ M calcein-AM is added into the medium. Preferably 5 to 10 μ M calcein-AM is used. Fluorescence is measured using a multi-well plate reader (Victor2, Wallac Oy, Finland) with following settings: Ex/Em = 485/530.

7. Defecation

The defecation of *C. elegans* is a recurrent event comprising of the following steps: pBoc, aBoc and expulsion. Defecation in nematodes such as *C. elegans* is 25 achieved by periodically activating a defined sequence of muscle contractions. These contractions are started in the anterior body wall muscles. At the zenith of the anterior body contractions the four anal muscles also contract. The four anal or enteric muscles are the two intestinal muscles, the anal depressor and the anal sphincter. In addition to this series of muscle contractions, specific neurons are also involved in the regulation 30 of defecation, including the motor neurons, AVL and DVB.

Method:

In order to construct a phenotypic profile, well-fed adults are typically examined after one day for constipation. The time between two pBocs is also scored.

5 The rate of defecation of *C. elegans* can also be quantitatively measured using an assay based on the following principles:

The rate of defecation of nematodes such as *C. elegans* can be easily measured using a marker molecule which is sensitive to pH, for example the fluorescent marker BCECF. This marker molecule can be loaded into the *C. elegans* gut in the form of the precursor BCECF-AM which itself is not fluorescent. If BCECF-AM is added to 10 nematode culture medium in the wells of a multi-well plate the worms will take up the compound which is then cleaved by the esterases present in the *C. elegans* gut to release BCECF. BCECF fluorescence is sensitive to pH and under the relatively low pH conditions in the gut of *C. elegans* (pH<6) the compound exhibits no or very low fluorescence. As a result of the defecation process the BCECF is expelled into the 15 medium which has a higher pH than the *C. elegans* gut and the BCECF is therefore fluorescent. The level of BCECF fluorescence in the medium (measured using a multi-well plate reader on settings Ex/Em=485/550) is therefore an indicator of the rate of defecation of the nematodes.

20 **8. Fertility**

A wild-type adult hermaphrodite *C. elegans* lays about 8 eggs per hour.

Method:

25 The amount of eggs laid by 20 hermaphrodite *C. elegans* during at least 60 min is counted. The amount of eggs may be counted by simple visual inspection or using a FANS device, described above.

Example 6

Comparison of profiles within a library

30 (daf-4 belongs to two pathways)

Mutant worms have been profiled according to the general profile protocol. Table 4 shows a summary of the profile, also called fingerprints, of one mutation of the

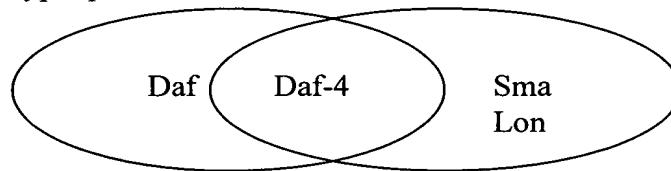
indicated genes. Entries are binary with empty fields indicating a phenotype (deviation from negative control, here wild-type) not found assuming that it could have been measured. Any other entry including comments or quantitative data is read as measured phenotype in this binary scheme and indicated by *.

5 The table lists only phenotypes that do have a positive entry, not necessarily complete, leaving pages of empty fields alongside and arranged according to a particular enquiry. The upper half consists of the hierarchical categories dauer formation phenotypes" and "body shape phenotypes" as well as their relevant sub-
10 phenotypes. The lower part consists of a set of hierarchically unrelated phenotypes subsumed under the enquiry categories, "increased activity" and "decreased activity".
The complete list of characteristics is to be found in Table 1.

15 The point of including the lower part is to show the principle of recording all observed phenotypes, that they can be used to distinguish similar phenotypic profiles in detail and that they can be arranged in order to make comparisons. In this case it is seen that the dichotomy of long versus short body length does not correlate to the dichotomy of increased versus decreased activity.

20 The upper part shows 5 genes (i.e. a mutation in that gene) affecting dauer formation as well as 5 genes affecting body shape in a particular combination. A mutation in one gene, daf-4, is unique in sharing the characteristics of both phenotypic groups. The following picture illustrates the phenotypic overlap as found by comparing entries in the phenotypic profiles.

25



From this overlap a hypothesis of a mechanistic link can be put forward for daf-4. In this particular case the mechanistic link is confirmed by the molecular nature of the genes, which as far as known are all members of the TGF β pathway by sequence 30 similarity:

	daf-7 TGF β like ligand	dbl-1 TGF β like ligand
	daf-1 type I receptor	sma-6 type I receptor
	daf-4 type II receptor	daf-4 type II receptor
5	daf-3 SMAD	sma-2 SMAD
	daf-14 SMAD	sma-3 SMAD
		sma-4 SMAD

The DAF-4 protein probably acts as a type II receptor in both pathways. The similarity of phenotypic profiles allows one to hypothesize mechanistic relationships in 10 a manner analogous to sequence similarity of genes. For example a compound which induces the phenotypes: longer or shorter body length in combination with 2 or 3 of pale, thin and variable egg size, in worms exposed to it, is very likely to act on a protein of the TGF β pathway.

15

Table 4

Phenotype	<i>daf-1</i>	<i>daf-7</i>	<i>daf-3</i>	<i>daf-14</i>	<i>daf-4</i> <i>e1364</i>	<i>sma-2</i> <i>e502</i>	<i>sma-3</i> <i>e491</i>	<i>sma-4</i> <i>e729</i>	<i>lon-1</i> <i>e185</i>	<i>lon-3</i> <i>e2175</i>
Dauer formation	●	●	●	●	●					
Constitutive dauer	●	●	●	●	●					
Recovery defective	●	●	●	●	●					
body shape					●	●	●	●	●	●
short					●	●	●	●		
long									●	●
thin					●	●	●	●	●	●
pale					●	●	●	●	●	●
irregular egg size					●	●		●	●	●
increased activity					●		●	●	●	●
enhanced movement					●		●		●	

Phenotype	<i>daf-1</i>	<i>daf-7</i>	<i>daf-3</i>	<i>daf-14</i>	<i>daf-4</i> <i>e1364</i>	<i>sma-2</i> <i>e502</i>	<i>sma-3</i> <i>e491</i>	<i>sma-4</i> <i>e729</i>	<i>lon-1</i> <i>e185</i>	<i>lon-3</i> <i>e2175</i>
amplitude increased									•	
head movement enhanced							•	•	•	•
foraging behaviour increased					•			•		•
pharynx pumping enhanced						•		•		
constitutive pumping						•	•	•		
no egg retention									•	•
decreased activity						•				
lay still						•				
slow movement						•				
pharyngeal pumping reduced						•				

Example 7

Comparison of phenotypes induced by acetylcholine esterase inhibitors

Wild type *C. elegans* adults have been exposed to acetylcholine esterase inhibitors at various concentrations. The worms have been profiled over two generations, meaning four profiles have been generated. All phenotypes from the phenotype list are displayed that have been measured in this experiment. Two phenotypes “loopy head movement” and “body dragged by head” are shared by most of the esterase inhibitors. This is called phenotype activity relationship (PAR, by analogy to structure activity relationship SAR). The shared phenotypes are used to identify the action of a new compound. The unshared phenotypes are used to distinguish drugs or unravel side effects when these phenotypes are part of another PAR.

Tabl 5

Phenotypes	Physostigmine	Neostigmine	Ambenonium	Tacrine	Galantamine	Trichlorfon
Thin	X					
Lay still	X					
Erratic	X					
Weak kinker		X				
Jerky				X		X
Enhanced head movement						X
Loopy head movement	X	X		X(L1)		X
Body dragged by head	X	X				X
Irregular touch response	X	X				
Reduced brood size	(X)					X
Delayed growth						X

Example 8

Comparison of phenotypes of mutations in the acetylcholine neurotransmission

5 **pathway**

C. elegans adults and larval stages that are homozygous for the mutations *cha-1*, *unc-17*, *snt-1* and *cat-1* have been profiled, meaning fingerprints have been generated. All phenotypes from the phenotype list are displayed that have been scored in this experiment. The phenotypes “small”, “resistance to CHA inhibitors (Ric)”, “slow

10 pumping” and “slow growth” are shared. This is called phenotype activity relationship (PAR, in analogy to structure activity relationship SAR). The shared phenotypes are used to identify genes in a pathway. The unshared phenotypes are used to distinguish these genes or unravel further functions in parallel or new pathways when these phenotypes are part of another PAR. The fingerprint of *cat-1* is different because this
15 gene is involved in the dopamine pathway.

Tabl 6

Phenotype	<i>cha-1</i> ChAT (synthesis)	<i>unc-17</i> VchAT (ACh- transporter)	<i>snt-1=ric-2</i> Synaptotag min homolog	<i>cat-1</i> VMAT (monamine- transporter)
Coiler	X	X		
Small	X	X	X	
Slow growth	X	X	X	
Ric	X	X	X	
Slow pumping	X	X	X	
Jerky when backing	X			
Low ChAT level	X			X
Poor male turning				
Enhanced foraging behaviour				X
Enhanced foraging behaviour				X
Defecation defects				X
Shrinker-uncs				

Example 9

Method to profile an intervention (mutation, compound etc)

5 Profiling a mutation in the gene *unc-17* that affects transportation of acetylcholine.

In the literature this phenotype is described, concerning movement, body size and feeding, as severe coiler, being rather small and thin and has only slow, irregular pumping of the pharynx (Riddle et al., "C. elegans II" Cold Spring Harbor Laboratory Press, 1997). By systematically describing *unc-17* the resulting fingerprint unravels more details and new properties: Concerning movement, body size and feeding the phenotypes strong coiler, spiralling inwards posteriorly, curly jerky and moves better forward, being small have been profiled. In addition defects in the sensory system, defecation and reproductive system have been found, in detail: the touch response is gone, constipation, aberrant defecation cycle (aBoc) and egg laying defective (no egg retention).

Example 10

Method to add biological information to a particular phenotype

One phenotype of the mutation *unc-4* is “coiler” (looks like a snail). The fingerprint of *unc-4* adds for “coiler” the details “ventral side out” and “spiralling inwards posteriorly”. This occurs when a set of neurons that control the forward movement of the ventral part of the worm (VA2 - VA10) gets the same input than another set of neurons that controls the backward movement of the ventral part (VB2 - VB10).

In this case the ventral muscles get contradicting signals and only the dorsal muscles contract properly. The result is a coiler that has only the ventral side outwards. We explain most of the phenotypes as consequence of a mislead process, here synaptic input.

Example 11

Comparison of phenotypes induced by compounds acting on GABAergic neurotransmission

Wild-type *C. elegans* adults have been exposed to GABA agonists (Muscimol) and GABA antagonists (Ivermectin and Fipronil) at various concentrations. Worms have been profiled and the scored phenotypes are displayed as fingerprints.

In addition, two mutations in the GABAergic pathway have been profiled and compared with the compound induced phenotypes: *unc-25* encodes for the decarboxylase and *unc-49* encodes for a GABA receptor.

The phenotype “shrinker” is present in all fingerprints (see Table dark grey). This phenotype is used as marker or diagnostic phenotype to identify activity of a compound or gene in the GABAergic pathway. There are further phenotypes only shared by some compounds and mutants (see Table light grey). These phenotypes are used to build a phenotype activity relationship (PAR).

The shared phenotypes are used to identify the action of a new compound when “shrinker” cannot be used or to reveal more details on a compound action. For example, all compounds and *unc-25* fingerprints contain constipation phenotypes but not the fingerprint of *unc-49*, although GABA is used for the defecation process. This is coincident with earlier findings that the UNC-49 gene product is not required for

defecation.

These results may indicate the existence of another yet unknown GABA receptor in *C. elegans*. The unshared phenotypes are used to unravel toxic side effects or other mode of actions.

5

Table 7

Phenotypes	Phenotypes (tc 11)	Muscimol	Ivermectin	Fipronil	<i>unc-25</i>	<i>unc-49</i>
"Phenotypes"						
Pale		x		x		
Motionless (paralyzed) I		x	x			
<i>Nearly motionless</i>		x	x			
No movement but motion II		x			x	x
<i>Little movement</i>		x		x	x	x
Slow movement III		x		x	x	
Enhanced movement V						
Stiff rods		x	x			
Loose rods						
Rigid paralysis (hypercontracted)		x	x			
Flaccid paralysis (relaxed)				x		
Bent body, jerky body, abnormal				x	(x)	
Omega appearance				x		x
Enhanced foraging					x	
Shrinker before movement		x		x		
Shrinker		x	x	x	x	x
No pumping		x	x			
Weak pumping						
Pumping frequency reduced			x	x		
Pumping frequency enhanced		x				
Pumping irregular						
Constipation			x	x	x	
<i>Foregut filled/enlarged</i>				x		
<i>Hindgut weak constipated</i>			x	x	x	
<i>Hindgut strong constipated</i>					x	
Defecation cycle defective		x	x	x	x	
(time: pBoc)						
Weak expulsion					x	
No expulsion						x
No egg retention (12-cell stage)						

Weak egg laying defect (comma)		
Strong egg laying defect (pretzel)	x	x
Bloated worms	x	
Bags of worms	x	

Example 12

Definition of body shape phenotypes

Aberrations of the body shape of *C. elegans* can be the result of mutations in a vast amount of genes. These genes may be required directly for the formation of the hypodermis, the hydroskeleton and the correct patterning of the worm body plan, e.g., collagen or even-skipped. They could be involved in the control of growth or metabolism like genes of the TGF β pathway or genes required for feeding. Eventually, mutations in certain genes that cause primary defects, e.g., absence of head muscle, cause secondary defects in the body shape like dystrophy in the head region.

Body shape phenotypes are all visible or measurable deviations of the body shape, colour and content. Phenotypes are comparatively measured against wild-type (N2, Bristol strain) and scored as deviation of wild type in the corresponding developmental stage, sex and preparation. The scored phenotype comes with the percentage of worms positive for that phenotype within a population.

Table 8

Scientific definition of body shape phenotypes. The phenotypes listed in the left column are described and defined in the right column. Some phenotypes are derived from the classical worm jargon like "dumpy", which is still shorter than "short and thick worm".

PHENOTYPE	DEFINITION
Proportion abnormal	
Short	Body length less than wild type.
Long	Body length more than wild type.
Thin	Body diameter less than wild type.
Thick	Body diameter more than wild type.
Dumpy	Body length less but body diameter more than wild type.
Spindle-shaped	body diameter is more for only a restricted region of the body.

Head defects

Hypertrophy of the head	Regions of the head are thickened. This additional tissue is part of the head and enclosed by the hypodermis.
Extensions of head	Small hypertrophied regions of the head.
Notched head	Extensions, protrusions on the dorsal side of the head.
Hammer head	Extensions at the head tip resemble a hammer like appearance.
Dystrophy of the head	Regions of the head are thinned due to missing tissue.
Swollen	The head looks like a balloon.
Rounded	The tip of the head is rounded.
Tapering	The tip of the head is tapering.
Vacuoles only in head	Vacuoles visible in the head but not in the rest of the body.
Only head bent	The head is held most of the time in a bent position. In extreme cases the worm looks like a walking stick.
Autodecapitation	The head/body connection is thinner, which results occasionally in an autodecapitation due to a body wall muscle contraction.

Body defects

Scrawny	Worm is shorter, thinner, pale and sick.
Hypertrophy of body	Regions of the body are thickened. This additional tissue is part of the body and is enclosed by the hypodermis.
Extensions	Small hypertrophied regions of the body.
Humpback	Extensions, protrusions on the dorsal side of the body. The counterpart, extensions on the ventral side of the body, would be scored as "multi vulva" in the section "vulva". The distinction between a non vulva-like extension versus a vulva-like extension will be made with a high power microscope.
Truncated body	Part of the body is missing.
Withered body	Part of the body is thinned.
Twisted	Twisted body. The rotation along the anterior-posterior body axis can be seen by the twisted gut/gonad tube or because the vulva and the rectum are not orientated in the same (ventral) direction.
Fat	Worm is thicker and darker than wild type.
Pale	Worm is brighter than wild type.
Pale with dark spots	Worm is brighter than wild type and contains dark spots.
Clear	Worm is nearly transparent.
Full of vacuoles	Worm contains more vacuoles than wild type. Vacuoles have a darker or opal appearance and resemble little moon craters.

Fluid-filled	Liquid flows all over the body.
Poured out	Contents of the worm like the gonad is released through the vulva.
Burst	Dead worm with bursted body shape.

Tail defects

Only tail truncated	Blunt body end; whipe is missing.
Tail shape aberrant	Tail or tail whipe is kinked, shortened or thickened.
Knob-like	Tail whipe has knob-like structures.

Cuticle defects

Blistered	Fluid-filled transparent blisters separated by the hypodermis outside on the body. Clearly different from extensions.
Molting defective	More worms are caught in their old skin like the sloughing of a snake.

It is possible to score body shape phenotypes by image acquisition followed by image analysis. The advantage in the automation of the profiling procedure is the quantification of the strength of a phenotype or the presence of the phenotype in a population. A disadvantage is that the procedure for analysing an image for every possible phenotype may be more elaborate than simply scoring by eye. Furthermore, certain details are difficult to access by video analysis e.g., blister versus protrusions.

Table 10

10 List of scientific body shape phenotypes, together with their corresponding technical definitions, in terms of characteristics which can be comparatively measured relative to wild-type characteristics using automated measuring apparatus.

Scientific phenotype	Technical definition	Technical phenotype
Proportion abnormal		
Short	Body length less than wild type	Short
Long	Body length more than wild type	Long
Thin	Body diameter less than wild type	Thin
Thick	Body diameter more than wild type	Thick
Dumpy		<i>Disappears</i>
Spindle-shaped		<i>Disappears</i>

Head defects

Hypertrophied head	Total head volume has increased	Hypertrophied head
Extensions on head	Head will be subdivided in n trapezes (or n slices). The diameter of different trapezes can be compared pairwise. The deviation of the diameter can also be located to one side	Extensions on head
Notched head		Extensions only on one side
Hammer head		Extensions are pairwise
Dystrophied head	Total head volume has decreased	Dystrophied head
Swollen		<i>Disappears</i>
Rounded	In the tip trapeze the top diameter is increased	Rounded
Tapering	The diameter of the tip trapezes are decreased	Tapering
Vacuoles only in head		<i>Disappears</i>
Only head bent	The head is most of the time in a certain position that can be measured by an average angle between tip and head/body connection	Tip of head is more often in one position
Autodecapitation		<i>Disappears</i>

Example 13

Use of GFP in profiling *C. elegans*

A lot of features of *C. elegans* as described in Table 1 can be easily monitored, 5 either automatically by image analysis, microtiter plate readers, or visual means, e.g. by normal microscopy or by Nomarski microscopy. Some features of *C. elegans* are more difficult to visualize. For these characteristics transgenic animals expressing a marker gene are very useful. Moreover, even for characteristics that are rather easily to score, the use of a nematode expressing a marker gene, such as GFP, LacZ, or luciferase, 10 enhances the fingerprinting of *C. elegans*. The *C. elegans* can be a wild type, a mutant, or a strain subjected to a compound or environmental stress, or a combination of those.

C. elegans mutant *unc-23* has a fingerprint, which comprises “jerky movement”, “tend to coil”, “bent head” and “egl”. Expressing GFP in the muscle cells of the animal could result in identification and scoring of additional characteristics such as 15 “improperly folded muscles”, and/or “detached muscles in head region”, and/or “no muscles in head region”, and/or “defective muscle attachment”, and/or “vulva muscle defects” (data not shown).

Similarly, *C. elegans* mutant *unc-71* has a fingerprint which comprise “reduced movement”, “weak amplitude”, “strong kinker”, and “slightly egl”. When introducing GFP in the neurons of the animals no apparent extra fingerprint features were observed. A closer look at the neurons of this mutant worm revealed at least following extra phenotypes: “fasculation defects”, “VD/DC connection defects” (data not shown).

GFP-phenotypes are hence very important in allowing phenotypes which are not otherwise visible to be measurable with Nomarski or dissection microscopy. GFP-phenotypes are further important in the pinpointing of defects to certain tissues and cells, and moreover GFP-phenotypes are important in distinguishing between similar defects with different causes.

What is claimed is:

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